ENHANCING LACCASE ACTIVITY USING PRO-OXIDANTS AND PRO-DEGRADANTS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/318,294, filed on September 10, 2001.

FIELD OF THE INVENTION

The present invention relates to the enhancement of enzyme activity. More specifically, the present invention relates to pro-oxidants and pro-degradants that, in combination, are useful in enhancing the activity of enzymes having laccase activity, especially in the field of pulp bleaching.

BACKGROUND OF THE INVENTION

Paper pulp is typically processed from wood through the Kraft (and other) processes. The process produces a pulp with a dark brown color, mostly due to the presence of lignin and lignin derivatives. For many applications, the lignin has to be removed by a process known in the art as "bleaching." This is typically done commercially in several stages in pulp mills, wherein lignin is first oxidized and then removed from the pulp.

Recently, several research groups have been working with enzymes to biologically bleach pulp, referred to as "bio-bleaching." Bio-bleaching is a methodology whereby an enzyme is used to decrease the optical brightness and/or lignin content of pulp or paper. The standard measure of bleaching efficiency is "Kappa number." Enzymes that have most commonly been used include laccase, lignin peroxidase, and manganese peroxidase. An enzyme group that has received particular attention is the laccase family of enzymes, which are copper-containing enzymes that are known to be good oxidizing agents in the presence of oxygen. Laccases are found in microbes, fungi, and higher organisms.

For many applications, the oxidizing efficiency of a laccase can be improved through the use of a mediator, also known as an enhancing agent. Systems that include a laccase and a

mediator are known in the art as laccase-mediator systems (LMS). There are several known mediators for use in a laccase-mediator system. These include HBT (1-hydroxybenzotriazole), ABTS [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfinic acid)], NHA (N-hydroxyacetinilide), NHAA (N-acetyl-N-phenylhydroxylamine), HBTO (3-hydroxy-1,2,3-benzotriazin-4(3H)-one), and VIO (violuric acid). In addition, there are several compounds containing NH-OH or N-O that have been found to be useful as mediators.

A major limitation of this methodology in the commercial context is the cost and the efficiency of the mediators used. It is critical therefore to discover additional mediators.

SUMMARY OF THE INVENTION

The present invention provides two-component mediators, wherein one component serves as a pro-oxidant and the other component serves as a pro-degradant and booster. When used in combination with a laccase, the two-component mediators enhance the bleaching of pulp. Thus, the invention provides a two-component mediator for use in a laccase-mediator system, the two-component mediator comprising (i) a pro-oxidant and (ii) a pro-degradant.

The invention also provides a composition comprising an oxidative enzyme, a pro-oxidant, and a pro-degradant The invention also provides a process for oxidizing a substrate that comprises treating the substrate with an oxidizing enzyme, a pro-oxidant, and a pro-degradant. Further, the invention provides a process for bleaching a lignin-containing material that comprises treating the material with an oxidative enzyme, a pro-oxidant, and a pro-degradant.

The process of the invention can further include the step of adding an oxidizing agent. In one embodiment, the oxidizing agent is at least one of air, oxygen, and hydrogen peroxide.

In one embodiment, the pro-oxidant is ascorbic acid, ascorbate, salicylic acid, salicylate, nicotinic acid, nicotinate, a hardwood black liquor, a softwood black liquor, ligno-organosolv,

lignin sulfonate, or a mixture thereof. In another embodiment, the pro-degradant is urea, thiourea, sulfamic acid, sulfamide, guanidine, methylsulfonic acid, or a mixture thereof.

DETAILED DESCRIPTION OF THE INVENTION

The present invention discloses the novel use of two components as mediators to enhance the bleach efficiency of an enzyme exhibiting laccase activity. It has been discovered that, although the use of a pro-oxidant or a pro-degradant alone as described herein is not necessarily useful as a mediator, the combination of a pro-oxidant or a pro-degradant together exert synergistic effects and produce better bleach efficiency for paper pulp.

For ease of reference and without loss of generality, one component is called a "pro-oxidant." Examples of the pro-oxidant include ascorbic acid, ascorbate, salicylic acid, salicylate, nicotinic acid, nicoliante, hardwood black liquor, softwood black liquor, ligno-organosolv, lignin sulfonate, and mixtures thereof. Compounds with related structures or structural analogs may also be used. The pro-oxidants are active mediators in the discoloration of a dye, e.g., Chicago Blue. However, these may or may not have efficiency as mediators for pulp bleaching.

For ease of reference and without loss of generality, the second component is called a "pro-degradant." Examples of the pro-degradant include urea, thiourea, sulfamic acid, sulfamide, and guanidine. Compounds with related structures or structural analogs may also be used. The pro-degradants are often not active mediators, even in the discoloration of a dye, e.g., Chicago Blue. In pulp bleaching tests with laccase, they show either negative results or slightly positive results.

It has been discovered that when a pro-oxidant and a pro-degradant are added together with a laccase enzyme, some combinations are capable of a large decrease in the Kappa number. The enzymes exhibiting laccase activity include the laccase enzymes of enzyme classification EC 1.10.3.2, the catechol oxidase enzymes of enzyme classification EC 1.10.3.1, the

monophenol monooxygenase enzymes of enzyme classification EC 1.14.99.1, and the bilirubin oxidase enzymes of enzyme classification EC 1.3.3.5. The EC (Enzyme Commission) number is based upon the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB).

The laccase of this invention may be derived from microbial, fungal, or other sources. It may furthermore be produced by a recombinant method, such as cultivating a host cell transformed with a recombinant DNA vector which includes a DNA sequence encoding the laccase (and DNA sequences encoding functions that permit the expression of laccase DNA sequence) in a culture medium under conditions that permit the expression of the laccase, and recovering the enzyme from the culture.

While the mediators are developed specifically for pulp bleaching, the same mediator mixtures and laccase enzyme(s) can be used for other applications, including treatment of pulp waste water, de-inking, industrial color removal, bleach for laundry detergents, oral care teeth whiteners, and as catalysts or facilitators for polymerization and oxidation reactions.

Another aspect of the invention provides a process for oxidizing a substrate that comprises treating the substrate with a composition comprising an oxidizing enzyme and a pro-oxidant and a pro-detergent. The pro-oxidant and pro-detergent can be selected from the above described pro-oxidants and pro-detergents.

The oxidative enzyme is a laccase, a catechol oxidase, a monophenol monooxygenase, a bilirubin oxidase, or a mixture thereof. The process further comprises adding a hydrolase, such as xylanase.

The pro-oxidants and pro-detergents may each be individually present in concentrations of from about 0.01 micromolar to 1000 micromolar, more preferably from about 0.1 micromolar to about 250 micromolar and most preferably from about 0.5 to about 100 micromolar.

The enzyme is used in amounts of from about 0.1 to 400 units (defined in Examples using ABTS as substrate) for 1 g dry pulp, more preferably from 1 to 200 units and even more preferably from about 10 to 100 units and most preferably from 20 to -50 units.

The process of the invention can further include the step of adding an oxidizing agent, such as at least one of air, oxygen, and hydrogen peroxide.

One embodiment of the invention provides a process for bleaching a lignin-containing material that comprises treating the material with an oxidative enzyme and a pro-oxidant and a pro-detergent. In this aspect of the invention, the a pro-oxidant and a pro-detergent may each be separately present in an amount of from about 0.1% to about 15% based on the weight of the dry lignin containing material, more preferably from about 0.1% to about 10% and even more preferably from about 0.5% to about 5% and most preferably from about 1% to about 4 %. The oxidative enzyme is a laccase, a catechol oxidase, a monophenol monooxygenase, a bilirubin oxidase, or a mixture thereof. In another embodiment, the process further comprises adding a hydrolase, such as xylanase.

One example of a lignin containing material is wood pulp. The process for bleaching a lignin-containing material can further include the step of adding an oxidizing agent, such as at least one of air, oxygen, and hydrogen peroxide.

EXAMPLES

Laccase Enzyme Assay

In the examples, two *Aspergillus* laccases have been used, both from Novozymes A/S (Denmark). NovoSample 51002 works best at pH 4-5 while NovoSample 51003 works best at

pH 5-6. Enzyme dosage has been found to have an effect on bio-bleaching. For example, 0.1 ml of the 51003 laccase gives a modest Kappa number reduction when HBT is used, and a huge reduction when ABTS is used.

The specific activity was determined using ABTS (0.5 mM) as substrate. One unit of activity is equal to the umol of the oxidized product from ABTS per min per mg protein at pH 6.0 at 23 °C. The extinction coefficient of the oxidized ABTS is: ε(max) at 420 nm=36,000M-1cm-1.)

Alternatively, the activity of laccase (NS51003) was determined using syringaldazine as substrate. In this case, one unit of activity is equal to the change of 0.001 UV absorbance at A530nm per minute per ug protein in 2 ml of 100 mM, pH 5.5 potassium phosphate buffer, and 0.5 ml of 0.25 mM syringaldazine in methanol at 23 °C.

Kappa Number

Delignification of the pulp was measured as the change in Kappa number according to TAPPI method T236 cm-85. Briefly, a known mass of paper pulp (containing lignin) was reacted with an excess of potassium permanganate in acid solution for a specified period of time to oxidize the lignin. After the reaction, the residual permanganate was determined by titration. The Kappa number was defined as the volume (ml) of 0.1N potassium permanganate consumed by 1 g of moisture-free pulp in 0.5N sulfuric acid after a ten-minute reaction time at 25 °C under conditions such that one-half of the permanganate remains unreacted. A linear relationship with the lignin content of the pulp and the measurement of the Kappa number has been done on samples as low as 300 mg of pulp.

Pulp Bleaching

A softwood Kraft pulp, Kappa number 31.0, was treated with a laccase (NS51003) under the following conditions:

Enzyme dosage	45 units /g pulp
pH	5.5
Temperature	50°C
Reaction time	16 hours
Pulp consistency	2%

The dried pulp was added to 80 ml of 50 mM phosphate, pH 5.5, and disintegrated in a blender. The pulp was then transferred to a 500-ml conical flask and the blender was washed with 20 ml of the same buffer. The washed buffer and the pulp were combined. The mediator was added at 1-4% (w/w, based on the dry pulp) followed by the addition of the laccase. The pH of the pulp mixture was adjusted to 5.5 if needed. The flask was covered with an aluminum foil with holes punched through and incubated at 50 °C for 16 hrs on a rotary shaker at 200 rpm.

After the enzymatic treatment, the pulp mixture was filtered through a Buchner funnel and the pulp was washed with water. The pulp from the control experiment was treated under the same pH and temperature conditions as described above. The washed pulp was then treated with an alkaline solution under the following conditions:

Pulp	2 g
Water	200 ml
NaOH	240mg
H_2O_2 (30%)	400ul
Temperature	70°C
Reaction time	3 hours

The filtered pulp was repulped in the alkaline solution and incubated at 70 °C for 3 hrs. The pH of the pulp mixture should be between 11.7-12.00 during the entire treatment. After the treatment, the pulp mixture was filtered through a Buchner funnel and the pulp was washed with water extensively and then dried in a hood overnight.

The delignification of the pulp was measured as the change in the Kappa number according to TAPPI method T236 cm-85.

The following examples are illustrative of the present invention, and are not intended to be construed in any way as limiting the scope of the invention.

Example 1 - Chicago Blue Assay of Pro-Oxidants and Pro-Degradants

In this example, the pro-oxidants and pro-degradants were tested individually as mediators to enhance laccase-catalyzed bleaching of Chicago Blue in solution. The Chicago Blue Dye, also known as Direct Blue 1 or DB1, is a commonly used assay for the oxidative efficiency of laccase. The Chicago Blue Dye is fully described by Schneider et al. in U.S. Patent No. 5,885,304, which is hereby incorporated by reference.

Each of the compounds (i.e., potential mediators) was dissolved in water, or in ethanol if the potential mediator was not water soluble, and then mixed with a phosphate buffer and a Chicago Blue solution. A solution of a laccase was added to make up 1 ml of the final solution, containing 20 uM mediator, 20 mM buffer at pH 5.5 or 7.0, 0.1-1% laccase (v/v) and Chicago Blue solution, with absorbance at A610 nm between 0.6 to 0.8. The change in the absorbance at A610 nm was measured immediately using a UV-VIS spectrophotometer (UV-1201, Shimadzu Scientific Instruments) after the enzyme was added. The decrease in absorbance was recorded at 30-second intervals for 5 minutes and was used to estimate the efficiency of the mediator.

The following are the results from the bleaching experiments of Chicago Blue:

Mediator (0.1umole/ml)	Usage (ugram)	ΔmA610 (3 min) at pH 5.5	ΔmA610 (3 min) at pH 7.0
none		0	0
ABTS	10	445	341
Lignin organosolv	20 ug/ml	390	265
Ascorbic acid	20 ug/ml	6	2
Nicotinic acid	20 ug/ml	0	0
Urea	20 ug/ml	1	0
Pine black liquor	100 (14%)	26	18
Lignosulfonic acid	25 ug/ml	24	-
Salicylic acid	20 ug/ml	11	-

Examples 2-6 - Pulp Bleaching Using Pro-Oxidant And Pro-Degradant

In these examples, we used the pulp bleaching as described above, laccase as the oxidative enzyme, a pro-oxidant (as mediator 2), and a pro-degradant (as mediator 1).

Example	laccase	mediator 1	mediator 2	pulp	Decrease in Kappa
2	0	0	0	2 g	0
3	100 ul	0	0	2 g	2.6
4	100 ul	urea, 87 mg	0	2 g	2.6
5	100 ul	0	ascorbate, 20 mg	2 g	4.8
6	100 ul	urea, 88 mg	ascorbate, 79 mg	2 g	7.1

The data above clearly indicate that urea is barely active in enhancing bleaching. However, in the presence of a pro-oxidant, a noticeable decrease in Kappa number was obtained.

Examples 7-13 - Pulp Bleaching Using Pro-Oxidant And Pro-Degradant

In these further examples, we used the pulp bleaching as described above, laccase as the enzyme, a pro-oxidant (as mediator 2), and a pro-degradant (as mediator 1). In the following table, SA = salicylic acid, AA = ascorbic acid. The decrease in Kappa number is the difference in the Kappa numbers of the corresponding experiments with and without mediators under the same experimental conditions used for the pulp tests.

Example	Laccase (ml)	Mediator 1	Mediator 2	Pulp (g)	Decrease in Kappa number
7	0.1	SA, 80 mg	Dicyandiamide, 80 mg	2	-4.7
8	0.1	SA, 80 mg	Melamine, 80 mg	2	-5.9
9	0.2	SA, 80 mg	sulfanilic acid, 80 mg	2	-4.0
10	0.15	SA, 80 mg	Sulfanilamide, 80 mg	2	-3.3
11	0.2	AA, 80 mg	Sulfanilamide, 80 mg	2	-4.1
12	0.1	AA, 80 mg	Melamine, 80 mg	2	-6.1
13	0.1	AA, 80 mg	Dicyandiamide, 80 mg	2	-6.1

It is to be understood that the above described embodiments are illustrative only and that modification throughout may occur to one skilled in the art. For example, a person of skill in the art will recognize that the mediators of the invention also include mediators which are functionally equivalent to the mediators specifically recited herein, such equivalents having minor structural variations such as the addition of a methyl or ethyl substituent or the formation of a methyl ester from a carboxylic acid. Accordingly, this invention is not to be regarded as limited to the embodiments disclosed herein.